REMARKS

Upon entry of the Amendment, claims 1-14 are all the claims pending in the application. Claims 11-14 have been withdrawn. Claims 1, 3, 4, and 8 have been amended. The amendment to claim 1 is supported in the specification, such as on pages 59-60. The amendments to claims 3 and 4 have been made, in view of the amendment to claim 1. The amendment to claim 8 is supported in the specification, such as on page 45 and pages 46-47. Therefore, no new matter has been added.

I. Specification

The disclosure is objected to allegedly for including a hyperlink language.

Applicants have accordingly amended the disclosure.

II. Claim Rejections - 35 U.S.C. § 112

(A) Claims 1-10 are rejected under 35 U.S.C. § 112, second paragraph, as allegedly being indefinite.

Applicants respectfully traverse.

The Examiner asserts that the phrase "hybrid-sensor kinase" as recited in claim 1 is unclear. The Examiner asserts that the difference between a "hybrid-sensor kinase" and an osmosensing histidine kinase is unclear.

The specification describes that a hybrid-sensor kinase is involved in signal transduction pathway that involves three proteins. *See* page 13 of the specification. The specification describes that a hybrid-sensor kinase is composed of an input region, a histidine kinase region, and a receiver regions at the C-terminal end. *Id.* at 12. In the signal transduction pathway, a

phosphate may be transferred from a hybrid-sensor kinase (a sensor) to a response regulator via an intervening phosphotransmitter protein.

Further, the specification describes that an "osmosensing histidine kinase having no transmembrane region" refers to an osmosensing protein which has a repeat sequence region of amino acid sequences, a histidine kinase region and a receiver region, but no transmembrane region. *See* pages 26-27 of the specification. Each repeat is composed of about 90 amino acids and shares amino acid sequence homology with the other repeats.

The specification describes the difference between the hybrid-sensor kinase and the osmosensing histidine kinase having no transmembrane region. *See* page 24 of the specification. In contrast to the input region of the hybrid-sensor kinase, the osmosensing histidine kinase having no transmembrane region. The input region of many hybrid-sensor kinases include a transmembrane region. *See* page 13. Instead of the transmembrane region, the osmosensing histidine kinase having no transmembrane region includes a repeat sequence region.

(B) Claim 8 is rejected under 35 U.S.C. § 112, second paragraph, as allegedly being indefinite.

The Examiner asserts that the phrase "derived from" is unclear.

Applicants have accordingly amended claim 8.

III. Claim Rejections - 35 U.S.C. § 102

Claims 1-3 and 5-10 are rejected under 35 U.S.C. § 102(b), as allegedly being anticipated by Cui *et al.* "An osmosensing histidine kinase mediates dicarboximide fungicide resistance in

Botryotinia fuckeliana (*Botrytis cinerea*)," Fungal Genetics and Biology, 36 (2002) 187-198 ("Cui").

Claim 1 presently recites that a cell is a bacterial cell, yeast cell, or a plant cell.

In contrast, Table 6 of Cui discloses *B. fuckeliana* resistant mutant strains. *B. fuckeliana* is not a bacterial cell, yeast cell, or a plant cell. *B. fuckeliana* is a filamentous fungi. *See, e.g.*, pages 187-188 of Cui. In this regard, Cui fails to anticipate the transformed cell recited in claim 1.

Claims 2-3 and 5-10 depend directly or indirectly from claim 1. In this regard, Cui fails to anticipate claims 2-3 and 5-10 for at least the same reasons as claim 1.

IV. Claim Rejections - 35 U.S.C. § 103

Claim 4 is rejected under 35 U.S.C. § 103 as allegedly being unpatentable over Cui.

Claim 4 indirectly depends from claim 1. Claim 1 presently recites that a cell is a bacterial cell, yeast cell, or a plant cell.

Referring to page 4 of the Office Action, it is the Examiner's position that a person of ordinary skill in the art would have been motivated to introduce *Dafl* into a *S. cerevisae* host cell either lacking a *Sln* gene or having a less active *Sln* gene. As described above, Cui is deficient in that it fails to describe or teach that the cell is bacterial cell, yeast cell, or plant cell. The Examiner asserts that Cui teaches that the histidine kinase pathway in *B. fuckeliana* and *S. cerevisae* are very similar pathways involving MAP kinases and transcription factors.

Cui proposes that *Dafl* encodes the first enzyme in an osmotic pathway in *B. fuckeliana* that operates in a similar manner to that in budding yeast. *See*, left column, page 195 and Figure

6. Cui teaches that *Bos1* gene of *B. fuckeliana* encodes a protein that exhibits characteristic HK features, including the conserved H-, X-, N-, D-, F-, and G-boxes. *See*, page 191, left column. Figure 3 of Cui shows that amino acid sequence homology between a protein encoded by the *Bos1* gene and a protein encoded by the *Sln1* gene is restricted to short conserved regions encompassing the phosphorylated histidine and receiver aspartic acid residues. Cui teaches that the protein encoded by *Bos1* gene of *B. fuckeliana* possesses six 90-amino acid repeat motifs near the N-terminus thereof. *See*, page 191, left column. Cui also teaches that the N-terminus shares homology with bacterial soluble sensory transducers and there is no transmembrane domain within the predicted protein, indicating that the protein is localized in the cytoplasm. *Id.* Cui teaches that wild-type *B. fuckeliana* is sensitive to antifungal compounds such as dicarboximide.

Applicants respectfully submit that a person of ordinary skill in the art would not have been motivated to introduce *Daf1* into a bacterial cell, yeast cell, or plant cell. As described in more detail below, the rejection amounts to an "obvious to try" standard, which is the incorrect standard for patentability. MPEP § 2145 (X)(B). Cui, at best, provides a general approach without giving any direction as to which of many possible choices is likely to be successful. Further, there is no reasonable expectation of success that the *B. fuckeliana* protein encoded by the *Daf1* gene would operate in an osmotic response pathway in *S. cerevicia*. MPEP § 2143.02.

The *Sln1* gene from *S. cerevisae* encodes a histidine kinase having transmembrane regions near the N-terminus and having no repeat motifs. Wild-type *S. cerevisae* is also not sensitive to antifungal compounds. Further, the amino acid sequence homology in Figure 3 of

Cui is evidence that a person of ordinary skill in the art would not have been motivated to introduce the *Daf1* gene into a *S. cerevisae* host cell either lacking a *Sln* gene or having a less active *Sln* gene.

Applicants submit herewith a copy of Nagahashi, *et al.* "Isolation of *CaSLN1* and *CaNIK1*, the genes for osmosensing histidine kinase homologues, from the pathogenic fungus *Candida albicans*," Microbiology (1998), 144, 425-432 ("Nagahashi").

Nagahashi is evidence that a person of ordinary skill in the art would not have reasonably expected the *Daf1* gene to operate in a *S. cerevisae* host cell either lacking a *Sln* gene or having a less active *Sln* gene. Nagahashi teaches producing *SLN1* deficient *S. cereviciae* cells harboring a *CaNIK1* gene in a multicopy plasmid. *See* page 430, right column. The protein encoded by the *CaNIK1* gene ("CaNikp") is from *Candida albicans*. CaNikp (1) has regions that are related to the sensor kinase and response regulator domains of two-component histidine kinase systems, (2) contains five repeats of about 90 amino acids with the N-terminal half thereof, and (3) lacks any apparent transmembrane domain. *See*, page 427, right column to page 430, left column. Nagahashi teaches that the *SLN1* deficient *S. cerevisiae* cell harboring the *CaNIK1* gene cannot grow. *See* page 430, right column. In view of its inability to grow, Nagahashi proposes that *CaNIK1* is functionally distinct from *S. cerevisiae SLN1* and that *CaNIK1* may not act in the same pathway thereof. *Id.* As *S. cerevisiae* and *Candida albicans* are both forms of yeasts, Nagahashi also teaches that there may be a divergence in osmosensing signal transduction mechanisms in yeasts. *See* page 431.

In this regard, similar to *CaNIK1*, a person of ordinary skill in the art would not have reasonably expected *Daf1* to operate in a *S. cerevisae* host cell either lacking a *Sln* gene or having a less active *Sln* gene. Cui does not provide teachings particular enough that would motivate a person of ordinary skill in the art to select *Daf1* or that would provide a reasonable expectation of success.

Thus, Claim 4 is not obvious at least by virtue of its dependence from claim 1.

In view of the above, reconsideration and allowance of this application are now believed to be in order, and such actions are hereby solicited. If any points remain in issue which the Examiner feels may be best resolved through a personal or telephone interview, the Examiner is kindly requested to contact the undersigned at the telephone number listed below.

The USPTO is directed and authorized to charge all required fees, except for the Issue Fee and the Publication Fee, to Deposit Account No. 19-4880. Please also credit any overpayments to said Deposit Account.

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Isolation of *CaSLN1* and *CaNIK1*, the genes for osmosensing histidine kinase homologues, from the pathogenic fungus *Candida albicans*

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Recent studies have revealed that fungi possess a mechanism similar to bacterial two-component systems to respond to extracellular changes in osmolarity. In *Saccharomyces cerevisiae*, Sln1p contains both histidine kinase and receiver (response regulator) domains and acts as an osmosensor protein that regulates the downstream HOG1 MAP kinase cascade. *SLN1* of *Candida albicans* was functionally cloned using an *S. cerevisiae* strain in which *SLN1* expression was conditionally suppressed. Deletion analysis of the cloned gene demonstrated that the receiver domain of *C. albicans* Sln1p was not necessary to rescue *SLN1*-deficient *S. cerevisiae* strains. Unlike *S. cerevisiae*, a null mutation of *C. albicans SLN1* was viable under regular and high osmotic conditions, but it caused a slight growth retardation at high osmolarity. Southern blotting with *C. albicans SLN1* revealed the presence of related genes, one of which is highly homologous to the *NIK1* gene of *Neurospora crassa*. Thus, *C. albicans* harbours both *SLN1*- and *NIK1*-type histidine kinases.

Keywords: *Candida albicans*, cloning, osmosensor, two-component system, histidine kinase

INTRODUCTION

Two-component systems which involve a phospho-relay from the histidine of the sensor kinase to the aspartic acid of the response regulator are widespread in bacteria (Bourret *et al.*, 1991; Stock *et al.*, 1991; Parkinson & Kofoid, 1992). Regulatory proteins similar to bacterial two-component systems are also found in some eukaryotes (Brown *et al.*, 1993; Ota & Varshavsky, 1993; Alex *et al.*, 1996; Chang *et al.*, 1993; Hua *et al.*, 1995; Wilkinson *et al.*, 1995). In *Saccharomyces cerevisiae*, Sln1p consists of an extracellular sensor, a kinase and a receiver domain (Ota & Varshavsky, 1993; Maeda *et al.*, 1994) and acts as an osmosensor protein (Maeda *et al.*, 1994). Under low osmolarity conditions, a specific histidine in the kinase domain is autophosphorylated. The phosphate moiety of this histidine is first transferred to a certain aspartic acid within the receiver domain and then via a phospho-relay to the downstream proteins Ypd1p and Ssk1p, leading to the shut off of the HOG1 MAP kinase cascade (Brewster *et al.*, 1993; Maeda *et al.*, 1994; Posas *et al.*, 1996). Histidine kinase activity and phosphorylation of Sln1p are essential for growth at low osmolarity because a mutation of either the autophosphorylating histidine or the receiver aspartic acid of Sln1p is lethal under these conditions (Maeda *et al.*, 1994). Increased osmolarity hampers the histidine kinase activity of Sln1p, which in turn activates downstream HOG1 MAP kinase (Brewster *et al.*, 1993; Maeda *et al.*, 1994; Posas *et al.*, 1996).

Involvement of a histidine kinase in an osmosensing pathway has also been reported in *Neurospora crassa* (Alex *et al.*, 1996). The predicted product of the *N. crassa NIK1* gene possesses two domains that are related to sensor histidine kinases and response regulators of bacterial two-component proteins (Alex *et al.*, 1996). Nik1p is an apparent cytoplasmic protein with six repeats of about 90 aa that may form a coiled-coil structure. Deletion of the *NIK1* gene caused aberrant

Abbreviation: 5-FOA, 5-fluoroorotic acid.

The GenBank/EMBL/DDBJ accession numbers for the sequences reported in this paper are AB006362 (*SLN1*) and AB006363 (*NIK1*).

hyphal morphology, a phenotype more prominent under high osmotic conditions (Alex *et al.*, 1996).

To address whether a histidine kinase osmosensing mechanism is conserved in other yeasts, we attempted the functional cloning of *Candida albicans SLN1*. In addition, probing a *C. albicans* genomic library with the *C. albicans SLN1* gene identified a gene that is highly related to *NIK1* of *N. crassa*. Thus, it seems that *C. albicans* has both Sln1p- and Nik1p-type histidine kinases which may allow adaptation to different osmotic conditions.

METHODS

Plasmid construction and yeast strain. A 700 bp *Hin*dIII–*Hin*dIII fragment that harbours the *tetO–HOP1* chimaeric promoter, UAS and URS, was excised from p97t (Nagahashi *et al.*, 1997), ligated with the DNA fragment containing the *hisG–URA3–hisG* module isolated from pMPY-ZAP (Schneider *et al.*, 1996) and cloned into Bluescript SKII+ to generate p97tZAP. Replacement of the cognate *SLN1* promoter with a tetracycline-controllable promoter (Nagahashi *et al.*, 1997) was achieved by the one-step gene replacement method (Baudin *et al.*, 1993; Schneider *et al.*, 1996) with slight modification. DNA fragments harbouring target sequences of the *SLN1* and tetracycline-regulated promoters and the *hisG–URA3–hisG* module were amplified by PCR using p97tZAP as a template and a pair of primers, 5′ CATCGAAAACAGCACGAACAAAAGCCAACTCAC-TACATTTTAGAACAGCTATGACCATG 3′ and 5′ TCC-AATTTTGATGGCAGGCCAAATCGCATTTGTATT-GGAATTCTTTTCTGAGATAAAG 3′. The resulting DNA fragment was transfected into an *S. cerevisiae* strain, YPH499 (*MATa ade2 his3 leu2 lys2 ura3*) that had been transfected with pINFAGAL4 and which constitutively expressed the *tetR–GAL4* fusion activator (Nagahashi *et al.*, 1997). After confirming the correct integration of the *tetO–HOP1* chimaeric promoter by PCR and Southern blotting, transfectants were selected by 5-fluoroorotic acid (5-FOA) and used for experiments. For convenience, we designated the above strain as Tet-SLN1. To determine the region of *CaSLN1* essential for complementing *ScSLN1*, deletion mutants of *CaSLN1* were cloned into the unique *Bam*HI site of YEp24T (Yamada-Okabe *et al.*, 1996) and transfected into another *S. cerevisiae* strain, A451 (MATα *can1 leu2 trp1 ura3 aro7*) in which the original *SLN1* locus was disrupted by *LEU2* but where episomal copies of *SLN1* in pYES2 (Invitrogen) were maintained under the control of the *GAL1* promoter. This strain, termed pYES-SLN1, was unable to grow in the presence of either 5-FOA or glucose as sole carbon source. Primers used to amplify *ScSLN1* were 5′ CCCGGGGAATTCATGCGAT-TTGGCCTGCCA 3′ and 5′ CCCGGGGAATTCTCATT-TGTTATTTTTCTT 3′. For *ScSLN1* disruption, the 2·3 kb *Pst*I–*Pst*I region of *ScSLN1* was replaced by *LEU2*.

Screening the *C. albicans SLN1* and *NIK1* genes. Tet-SLN1 cells were transfected with a *C. albicans* genomic DNA library and spread on plates containing 50 μg tetracycline ml^{-1}. After incubation at 30 °C for 3 d, colonies appeared on the plates, cells were collected and plasmid DNA was recovered from them. After a second screening, the essential region of the insert DNA that conferred tetracycline-resistant growth of Tet-SLN1 was determined by cloning each DNA fragment in YEp24T (Yamada-Okabe *et al.*, 1996) and in pRS416 (Stratagene).

For cloning the *NIK1* gene of *C. albicans*, a *C. albicans* genomic DNA library was screened with the 2·1 kb *Kpn*I–*Sal*I fragment of the *C. albicans SLN1* gene as probe. Hybridization was carried out under low stringency conditions in a buffer containing 0·25 M sodium phosphate (pH 7·2), 2 × SSC (1 × SSC contains 150 mM NaCl and 15 mM sodium citrate), 1% (w/v) bovine serum albumin, 1 mM EDTA, 0·1% (w/v) SDS and 25% (w/v) formamide at 37 °C for 12 h. Radiolabelling of DNA with [α-^{32}P]dCTP and DNA sequencing were carried out as described by Sambrook *et al.* (1989). Construction of *C. albicans* genomic DNA library was reported previously (Yamada-Okabe *et al.*, 1996).

Disruption of *CaSLN1*. The 2·3 kb *Kpn*I–*Kpn*I fragment of *CaSLN1* was cloned in pUC19 and the resulting plasmid digested with *Bal*I and *Sna*BI followed by ligation with a 3·8 kb *Bam*HI–*Xba*I fragment carrying the *hisG–URA3–hisG* module, generating pCASLN1U. Thus, the 0·6 kb *Sna*BI–*Bal*I region of *CaSLN1* was replaced by the *hisG–URA3–hisG* module in pCASLN1U. After pCASLN1U was linearized by digestion with *Puv*II, 10 μg DNA was transformed into *C. albicans* CAI4 (*ura3Δ::imm434/ura3Δ::imm434*) cells by the lithium acetate method (Sanglard *et al.*, 1997). Before and after a second round of transformation, the *URA3* gene was excised by 5-FOA as described previously (Mio *et al.*, 1996). Unless otherwise specified, *C. albicans* cells were cultured in YPD (1%, w/v, yeast extract; 2%, w/v, peptone; 2%, w/v, glucose) in the presence or absence of 1·5 M NaCl.

RESULTS

Functional cloning of the *C. albicans SLN1* gene

To isolate a *C. albicans SLN1* gene by functional cloning, we generated an *S. cerevisiae* strain in which *SLN1* expression was conditionally repressed. This strain, designated Tet-SLN1, grew normally in the absence of tetracycline, while its growth was severely impaired by the addition of 50 μg tetracycline ml^{-1}. Transfection of Tet-SLN1 with intact *S. cerevisiae SLN1* restored normal growth even in the presence of 50 μg tetracycline ml^{-1}, demonstrating that the growth defect of Tet-SLN1 caused by tetracycline was largely due to the repression of *SLN1* expression.

Tet-SLN1 cells were transfected with a *C. albicans* genomic DNA library that was constructed with a vector harbouring the *S. cerevisiae TRP1* gene and a 2 μ replication origin (Yamada-Okabe *et al.*, 1996) and transfectants were selected using 50 μg tetracycline ml^{-1}. From 10^4 independent colonies, three clones grew in the presence of 50 μg tetracycline ml^{-1}. The plasmid DNA was recovered from these clones and a restriction map of each insert DNA determined. Although the restriction maps of these three clones differed from each other, the map of clone 1 coincided with the pattern of a *C. albicans* genomic Southern blot that was obtained using the *S. cerevisiae SLN1* gene as a probe and this clone was analysed further.

Deletion analysis with this clone demonstrated that a 5·6 kb *Eco*RV–*Xho*I region was sufficient to rescue Tet-SLN1 cells in the presence of tetracycline (Fig. 1a). Sequencing of this region revealed that it contained a long ORF of 3·6 kb, with a coding sequence possibly

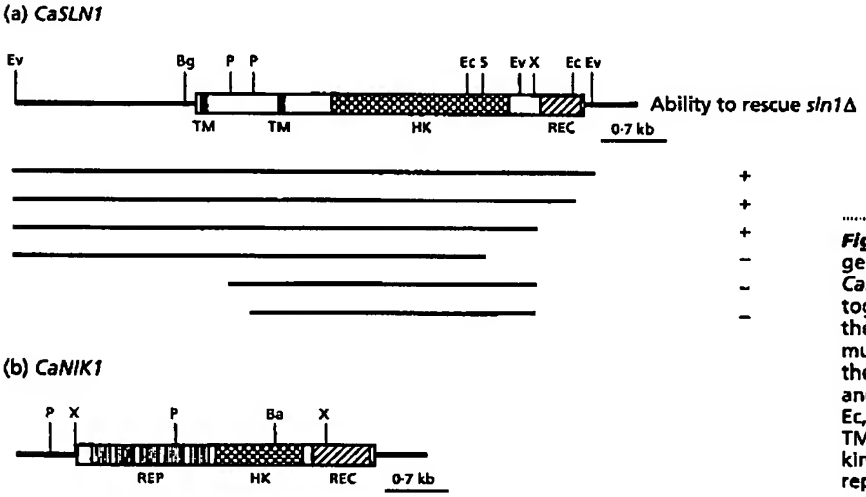

Fig. 1. Restriction maps of *C. albicans* genomic DNA fragments that contain *CaSLN1* (a) and *CaNIK1* (b) are illustrated together with the expected structures of their products. The ability of deletion mutants to rescue pYES-SLN1 cells (*sln1Δ*) in the presence of 5-FOA is indicated as '+' and '−', respectively. Ba, *Bam*HI; Bg, *Bgl*II; Ec, *Eco*RI; Ev, *Eco*RV; P, *Pst*I; S, *Sal*I; X, *Xho*I; TM, transmembrane domain; HK, histidine kinase domain; REC, receiver domain; REP, repeats of an approximately 90 aa motif.

extending further downstream. To obtain a clone containing the missing 3′ end of the ORF, we screened the same *C. albicans* genomic DNA library using a 0·5 kb *Sal*I–*Xho*I fragment as a probe. Sequencing of a clone containing the complete ORF revealed that the predicted product of the gene is a 150 kDa protein highly similar to *S. cerevisiae* Sln1p (37% identity) (Fig. 2a) and the gene was designated *C. albicans SLN1* (*CaSLN1*). To avoid confusion, the *S. cerevisiae SLN1* was termed *ScSLN1* in this study. Like ScSln1p, CaSln1p has no N-terminal signal sequence, but possesses two potential transmembrane helices in its N-terminal half (Fig. 3a). Sequence identity between CaSln1p and ScSln1p was remarkably high within the histidine kinase and receiver domains located in the middle of the protein and near the C-terminal end, respectively, and both the essential phosphorylating histidine at position 576 and receiver aspartic acid at position 1144 of ScSln1p are conserved in CaSln1p (Fig. 2a).

As described above, the initially isolated *CaSLN1* clone lacked the C-terminal receiver domain, suggesting that the receiver domain of CaSln1p is not crucial to complement a *ScSLN1* deletion. We confirmed this by making a series of deletion mutants and transfecting them into another *S. cerevisiae* strain, pYES-SLN1. Consistent with the results of the functional cloning of *CaSLN1*, the 5·6 kb *Eco*RV–*Xho*I fragment of *CaSLN1* that eliminates the receiver domain from CaSln1p still rescued pYES-SLN1 in the presence of 5-FOA or glucose, while further deletion from C terminus, destroying half of the probable ATP-binding site within the histidine kinase domain lead to loss of the ability to complement *ScSLN1* (Figs 1a and 4).

Disruption of *CaSLN1*

To study the function of *CaSLN1*, we generated the homozygous *casln1Δ* null mutant strain by means of the ura-blaster protocol (Fonzi & Irwin, 1993). Using this strategy, the first one-third of the histidine kinase domain, including the probable autophosphorylating histidine at position 519 of CaSln1p, was replaced by the *hisG* sequence of *Salmonella typhimurium* (Fig. 5a). The correct integration of the *hisG* sequence into the expected loci was confirmed by Southern blotting (Fig. 5b). Unlike *S. cerevisiae*, the homozygous *casln1Δ* null mutant of *C. albicans* grew under both normal and high osmotic conditions and sustained an ability to form hyphae. However, the homozygous null mutation, but not the hemizygous mutation of *CaSLN1* caused weak growth retardation in the presence of 1·5 M NaCl (Fig. 6a). Moreover, the homozygous *casln1Δ* null mutant cells elongated in the presence of 1·5 M NaCl (Fig. 6b). Similar morphological changes of the homozygous *casln1Δ* null mutants were also observed in the presence of 1 M sorbitol or 1 M KCl (data not shown).

The above results clearly demonstrate that *CaSLN1* is not an essential gene in *C. albicans* and may imply that *C. albicans* harbours other genes, possibly histidine kinases, to adapt to high osmolarity.

Cloning the *C. albicans NIK1* gene

In an attempt to search for other histidine kinase genes in *C. albicans*, we performed genomic Southern blotting with a part of *CaSLN1* DNA corresponding to the histidine kinase domain of CaSln1p and detected several discrete bands that were not derived from *CaSLN1* alleles. A genomic DNA library was again screened with the 2·1 kb *Kpn*I–*Eco*RI fragment of *CaSLN1* as a probe and a clone distinct from *CaSLN1* was obtained. This clone contained an ORF that could encode a 119 kDa protein highly similar to *N. crassa Nik1p* (50% identity) (Fig. 2b) and the gene was termed *C. albicans NIK1* (*CaNIK1*). Like *N. crassa* Nik1p, CaNik1p contains five repeats of about 90 aa within the N-terminal half (Figs 1b and 2b) and a hydropathy plot of CaNik1p lacks any apparent transmembrane domain (Fig. 3b). However,

(a)

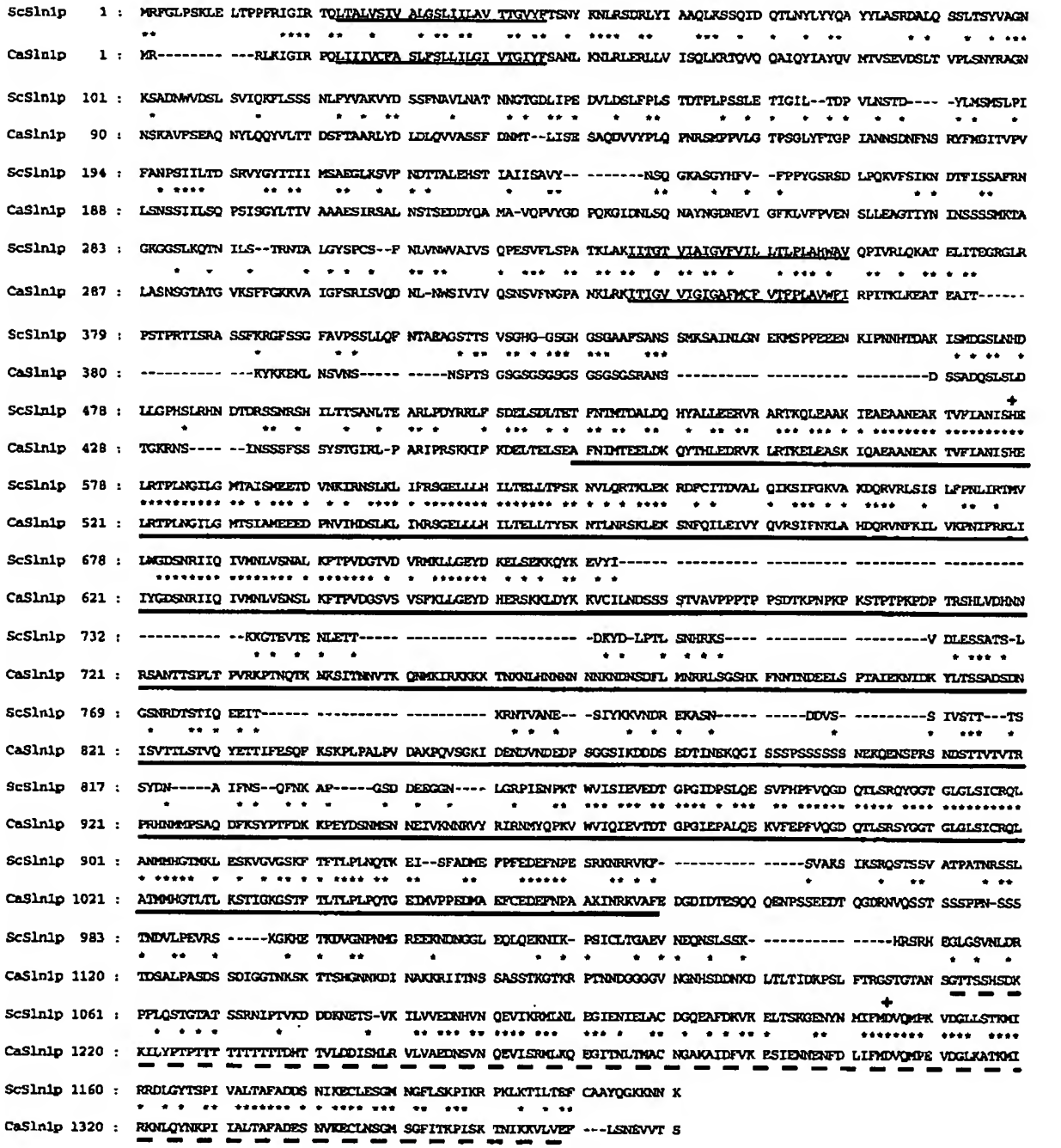

Fig. 2. For legend see facing page.

there are some structural differences between the two proteins. As mentioned above, *N. crassa* Nik1p contains six repeats of 90 aa that always start with tryptophan, but the first repeat in CaNik1p begins with glutamic acid and the fourth repeat observed in the *N. crassa* Nik1p is absent from CaNik1p (Fig. 2b). The regions from amino acid residues 490 to 641 and from 886 to 990 of CaNik1p share a high degree of sequence identity with the sensor

(b)

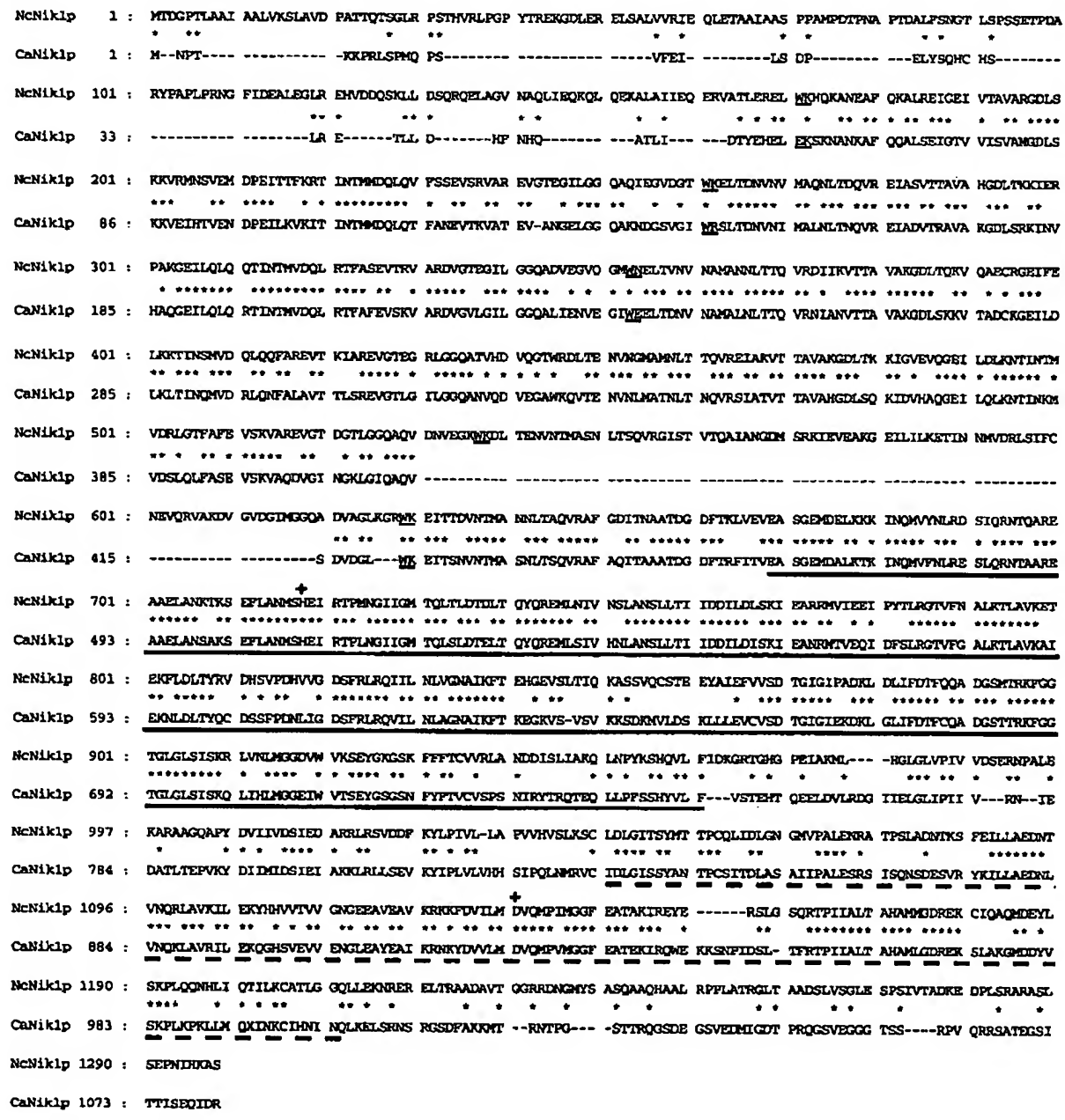

Fig. 2. (a) The amino acid sequence of *S. cerevisiae* Sln1p (ScSln1p) is compared with that of *C. albicans* Sln1p (CaSln1p). Identical amino acids between ScSln1p and CaSln1p are marked by asterisks. Probable transmembrane regions of ScSln1p and CaSln1p are double-underlined. (b) The amino acid sequence of *N. crassa* Nik1p (NcNik1p) is compared with that of *C. albicans* Nik1p (CaNik1p). Identical amino acids between CaNik1p and NcNik1p are marked by asterisks. The first two amino acids of each repeat of about 90 aa in NcNik1p and CaNik1p are double-underlined. Amino acid sequences were aligned using the BLAST and FASTA programs. Predicted histidine kinase and receiver domains are indicated by bold underlining and dashed bold underlining, respectively. '+' represents the positions of the histidine and aspartic acid residues that correspond to the autophosphorylated histidine and phosphorylated aspartic acid residues of ScSln1p. According to the report of Santos & Tuite (1995), the CTG codon is decoded as serine instead of leucine.

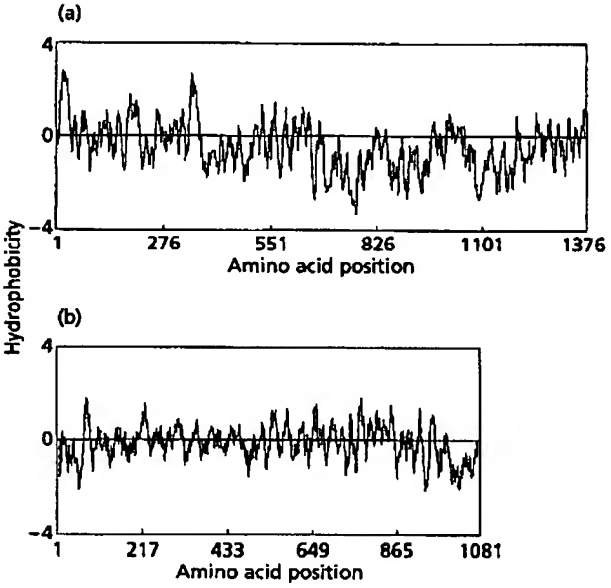

Fig. 3. Hydropathy plots of *C. albicans* Sln1p (a) and Nik1p (b) were calculated as described by Kyte & Doolittle (1982) using a window of 12 aa. It should be noted that two short domains with hydrophobicity indices of above 3 in CaSln1p (a) are considered as transmembrane helices.

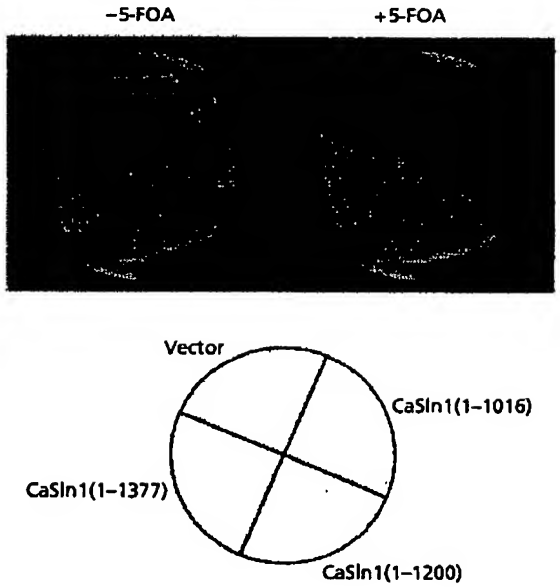

Fig. 4. Functional complementation of *ScSLN1* by *CaSLN1*. *S. cerevisiae* pYES-SLN1 cells were transfected with YEp24T (vector) or YEp24T harbouring *CaSLN1*, which can encode full length CaSln1p [CaSln1(1–1377], or C-terminally truncated CaSln1p [CaSln1(1–1200) and CaSln1(1–1016)]. The transfectants were spread on plates with (+) or without (−) 5-FOA and were incubated for 3 d. The receiver domain is deleted in CaSln1(1–1200) and the receiver domain and half of ATP-binding site are destroyed in CaSln1(1–1016).

kinase and response regulator domains of two-component systems, respectively. Sequence comparisons with *S. cerevisiae* Sln1p and the BarA protein of *Escherichia coli* implicate the histidine at position 510 and the aspartic acid at position 924 as sites of phosphorylation (Fig. 2b).

DISCUSSION

We have isolated and sequenced a *C. albicans* homologue of *ScSLN1*. Like ScSln1p, CaSln1p possesses extracellular sensor, histidine kinase and receiver domains. When expressed from a multicopy plasmid, *CaSLN1* overcame the growth defect of *S. cerevisiae* cells caused by the repression of *ScSLN1*. Since CaSln1p also shares significant sequence identity with the probable ATP-binding site within the kinase domain of ScSln1p, it should be able to autophosphorylate a histidine residue of the protein.

The receiver domain of CaSln1p was not necessary to rescue *sln1Δ S. cerevisiae* cells. However, the mechanism of the complementation of ScSln1p by C-terminally truncated CaSln1p is not clear. In *S. cerevisiae*, the phosphate moiety at the autophosphorylated histidine residue in the kinase domain of Sln1p is transferred to an acceptor aspartic acid residue in the receiver domain of the same protein, then to a histidine residue in the downstream Ypd1p and finally to an aspartic acid residue of the further downstream Ssk1p, with both the autophosphorylated histidine and receiver aspartic acid residues being essential for viability under low osmotic conditions (Posas *et al.*, 1996). One possibility is that CaSln1p can bypass the phospho-relay to the receiver domain and to Ypd1p, and can function by directly phosphorylating Ssk1p.

In addition to *CaSLN1*, another gene, *CaNIK1*, was isolated and sequenced. The product of *CaNIK1* is highly homologous to Nik1p of *N. crassa*, with regions that are highly related to the sensor kinase and response regulator domains of two-component systems. Although there is no apparent transmembrane helix in CaNik1p, we asked if CaNik1p has a similar function to ScSln1p. However, preliminary experiments did not support this hypothesis, because pYES-SLN1 cells harbouring *CaNIK1* in a multicopy plasmid were unable to grow in the presence of 5-FOA or glucose. This result implies that *CaNIK1* is functionally distinct from *S. cerevisiae SLN1* and that *CaSLN1* and *CaNIK1* may not act in the same pathway. In fact, sequence homology between CaSln1p and CaNik1p is restricted to short regions encompassing the phosphorylated histidine and receiver aspartic acid residues. However, we cannot yet rule out the trivial possibility that the *CaNIK1* gene failed to function in *S. cerevisiae*.

Unexpectedly, the homozygous *casln1Δ* null mutation

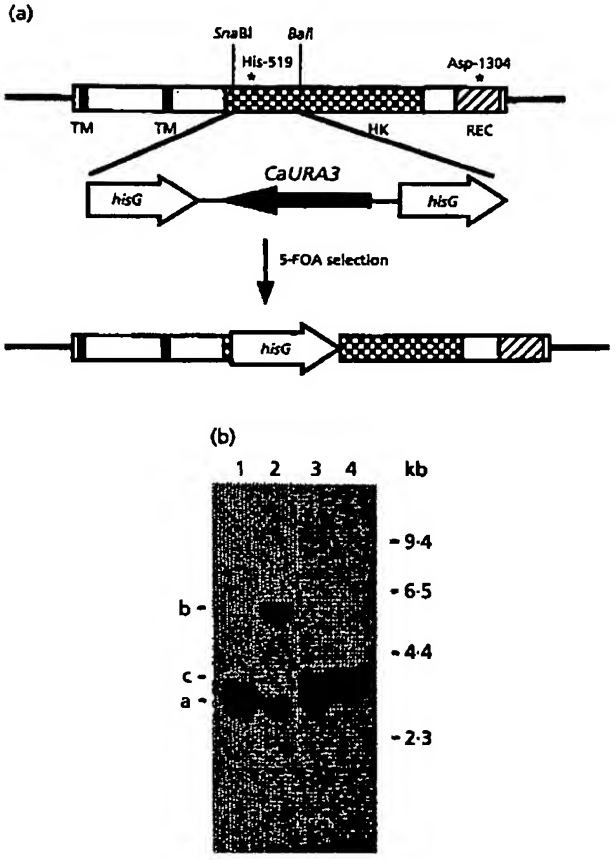

Fig. 5. Generation of the homozygous *casln1*Δ null mutant strain. (a) The strategy for disrupting *CaSLN1* is illustrated, with the *SnaI/BalI* region of *CaSLN1* replaced by the *hisG* sequences. (b) Southern blotting of *CaSLN1*. Twenty-five micrograms of genomic DNA from wild-type CAI4 (lane 1), the hemizygous *casln1*Δ mutant with *URA3* (lane 2), the hemizygous *casln1*Δ mutant without *URA3* (lane 3) and the homozygous *casln1*Δ null mutant (lane 4) was digested with *BglII* and *SalI*, fractionated on an agarose gel and hybridized with the 0·9 kb *BalI–SalI* fragment of *CaSLN1*. Bands derived from the *CaSLN1* allele, the *casln1*Δ::*hisG–URA3–hisG* allele and the *casln1*Δ::*hisG* allele are indicated by a, b and c, respectively.

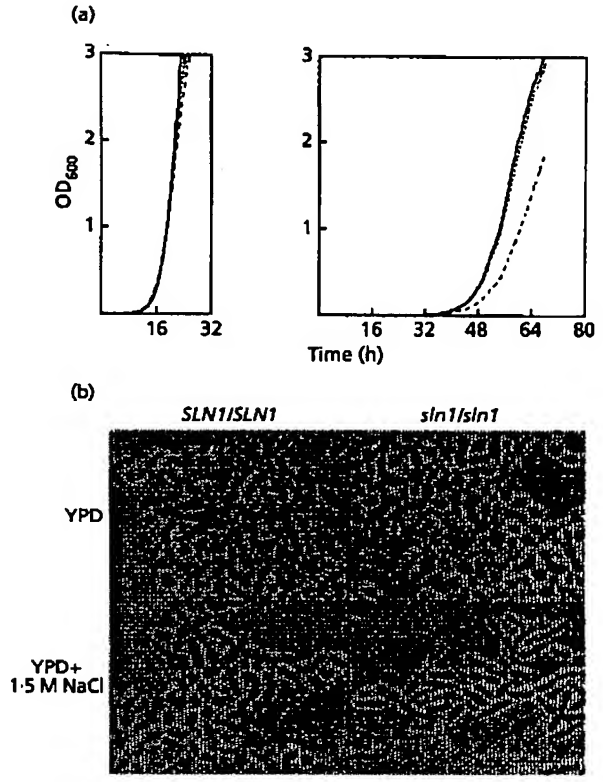

Fig. 6. Effects of the disruption of *CaSLN1* on growth and morphology. (a) Effects of high osmolarity on the growth of wild-type CAI4 (*SLN1/SLN1*) (——), the hemizygous *casln1*Δ mutant (*SLN1/sln1*Δ) (....) and the homozygous *casln1*Δ null mutant (*sln1*Δ/*sln1*Δ) (– – –). Cells of the indicated strains were cultured in YPD medium in the absence (left) or presence (right) of 1·5 M NaCl and the growth of the cells was monitored using a Biophotorecorder (Advantec). (b) Morphological change caused by the disruption of *CaSLN1*. Cells of wild-type CAI4 (*SLN1/SLN1*) and of the homozygous *sln1*Δ null mutant (*sln1*Δ/*sln1*Δ) were cultured in YPD medium in the absence (upper panels) or presence (lower panels) of 1·5 M NaCl. Photographs of cells from overnight cultures are shown. Bar, 10 μm.

was not lethal in *C. albicans*. The homozygous *casln1*Δ null mutant cells grew even under high osmotic conditions, but growth in the presence of 1·5 M NaCl was somewhat impaired by disruption of *CaSLN1*. This phenotype resembles that of the *nik1*Δ null mutant of *N. crassa* (Alex *et al.*, 1996) and also that of the *hog1*Δ null mutant of *C. albicans* (Jose *et al.*, 1996) and is suggestive of a CaSln1p function under high osmotic conditions. In contrast, the histidine kinase activity of ScSln1p is necessary under low osmotic conditions and is repressed under high osmotic conditions, leading to activation of the HOG1 MAP kinase in *S. cerevisiae*. Thus, it seems to be puzzling why a disruption of *casln1*Δ caused a growth defect at high osmolarity. Although further experiments, including the disruption of *CaNIK1*, are under way to address this question, the absence of a *NIK1* homologue in the *S. cerevisiae* genome suggests a divergence of osmosensing signal transduction mechanisms in fungi.

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